USE OF CRYSTALLINE TYPE A BOTULINUM TOXIN IN MEDICAL RESEARCH

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The various toxins produced by *Clostridium botulinum* are extremely potent neurotoxins. Type A toxin (one of the 6 recognized types) is easily produced in deep culture and the first to be obtained in a highly purified crystalline form. It is a high molecular weight simple protein (about 900,000) and dissociates under certain conditions of pH and ionic strength into a protein of about 150,000 molecular weight having the neurotoxin properties and another possessing hemagglutinating properties which appears very important in stabilizing the toxic portion of the molecule. The toxin has the specific physiological action of causing a presynaptic block by inhibiting in some manner the release of acetylcholine at the myoneural junction and producing a flaccid paralysis of the muscle which requires about three weeks or more for recovery.

The work of Scott (1) originally presented at the 84th Annual Meeting of the American Academy of Ophthalmology in San Francisco, California, 1979, and recently published in Ophthalmology (2) has taken advantage of this property of the toxin to treat strabismus in humans by injecting a small amount of toxin under carefully controlled conditions directly into the extraocular muscle pulling the eye out of

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alignment. The amount of toxin used in the treatment depends upon the condition of the patient. At the present time the toxin used for this treatment is an ultrafiltered preparation of 0.05 micrograms (µg) of crystalline toxin (116 mouse IP LD_{50}) lyophilized with human serum albumin and saline in small ampoules kept under vacuum. This preparation appears to be appropriate and reliable in every respect for medical use in the treatment of strabismus in humans. Although the specific toxicity of the crystalline toxin of 3×10^7 mouse IP LD_{50} per mg, or 1.5×10^3 LD_{50} for the 0.05 µg in an ampoule, drops more than one log during filtration and lyophilization, this drop is relatively constant from one preparation to another and close to 116 mouse LD_{50} remains in the ampoule. For treatment 0.64 ml or more, depending upon the dose to be given, of sterile saline is introduced aseptically into the ampoule to dissolve the toxin and a water clear solution is produced. A 0.1 ml of this solution, using an electromyrographic needle, is injected into the muscle. Experience gained by Scott indicates that about 1 mouse LD_{50} is a starting dose and this is repeated or increased according to the response of the patient. Upon recovery the muscle tends to stay in the proper position and corrected cases, now over 2 years old, have remained so. The maximum time of paralysis occurs 4 or 5 days following the injection, and then gradually diminishes, depending on the dose. The maximum correction of strabismus has been 20 degrees. The maximum follow-up following injection is 6 months. The results after the treatment of 43 cases in humans have been remarkably good and the simplicity of the treatment definitely makes it an alternative to surgery for the correction of strabismus. Details of the treatment are given in publications by Scott (2). One concern regarding this preparation is the presence of detoxified toxin in the presence of active toxin. One injection however would deposit less than one ng of detoxified toxin. This amount seems to be inconsequential and probably insufficient to ilicit any antibody production. At least detoxified toxin appears to have no observable effect when injected IP or IV into mice and Dr. Scott has observed no effects on humans.

Although the preparation described above is, for all practical purposes, satisfactory for the treatment of strabismus, the ideal preparation for this treatment, or for any other medical use of the toxin would be one in which the full toxicity was maintained during preparation and on long time storage.

Studies have been undertaken to accomplish the ideal preparation. One of the purposes of this paper is to describe some of the important problems regarding the nature

and properties of the toxin that are involved in its preparation for medical use; that is the use of the toxin as a drug. Botulinum toxin, like other proteins that possess biological activity, such as some enzymes, possesses its extreme toxicity due to its conformational structure (3,4). It is therefore detoxified in solution by heat, various chemicals, dilution to low concentrations, surface stretching and surface drying. To make a preparation suitable for medical use it was necessary to find means to preserve the toxicity and considerations were given to: (a) purity; (b) factors involved in making a reliable and stable preparation; (c) some data on dose response in animals; and (d) sterility of the preparation.

In regard to purity, the crystalline toxin, upon ultracentrifugation at pH 5.6 or below, is a homogeneous substance of constant composition and activity. From the time Lamanna (5) found that the crystalline toxin could be dissociated into toxin and hemagglutinin by treating with red blood cells at pH 7.3 there has been a question about the advisability of using crystalline toxin in physiological research because of the possible effects of the hemagglutinin on the action of the neurotoxin. The separation of the neurotoxin from hemagglutinin by physical means by others (6,7) has pointed out the marked instability of the neurotoxin without the hemagglutinin (8). It is believed that the hemagglutinin is dissociated from the neurotoxin in the body when consumed orally and that the neurotoxin only reaches the site of action. When a solution of the crystalline toxin is injected directly into a muscle both the toxin and hemagglutinin are present. The work of Scott (2) has not indicated any undue side effects of the hemagglutinin when the crystalline toxin was injected into the extraocular muscle. An important point regarding the use of the purified neurotoxin besides its instability is the fact that it cannot be prepared with constant composition and activity.

The stability of the toxin in a preparation or medical use and its long time storage without loss of toxicity is a very important factor if the dose is to be reliable. Crystals of the toxin are stable for several years when suspended in 0.9 M ammonium sulfate solution and refrigerated. Dispensing a suspension of such extremely toxic crystals into units of 10 ng is not practical and cannot be done accurately. Our studies therefore have been directed toward the development of a suitable medium for solution of the toxin that would retain the specific toxicity over a reasonable length of time, perhaps for 2 years. The specific toxicity of the crystalline toxin in solution is 3×10^7 mouse IP LD_{50} ± 10% per mg using the white mice available in

our laboratory. The specific toxicity varies with different kinds of mice and the conditions under which the assay is carried out. To get around this variation and consistently produce a uniform preparation the crystalline toxin must be measured by its extinction coefficient of 1.65 for one mg per ml at 278nm in a one cm light path and must have a 260nm to 278nm absorption ratio of 0.55 or less. Solutions of the toxin at concentrations of 2 mg or more per ml in 0.05 M acetate buffer at pH 4.2 are stable for long periods, but dilution to much lower concentrations results in its detoxification within a short period of time. Addition of other proteins such as gelatin or serum albumin greatly helps to prevent detoxification in dilute solution and the addition of gelatin is customarily made when diluting the toxin for the mouse assay. The addition of protein to a solution of the toxin at pH 4 to 4.5 was used for the establishment of a reference standard for the bioassay of toxin in foods and body fluids for the Food and Drug Administration (9). For this preparation a solution of 3X crystalline toxin in acetate buffer at pH 4.2 at a concentration of 2 to 4 mg per ml, accurately determined by its absorbance at 278nm, was diluted to a concentration of 100 ng per ml with a 0.05 M sodium acetate buffer at pH 4.2 containing 3 mg of bovine serum albumin and 2 mg of gelatin per ml. When 0.5 ml of this solution was sealed in 1 ml glass ampoules and stored at room temperature, the toxicity remained at the original level of 2500 LD_{50} for two years but gradually fell off to about 1000 LD_{50} or 50% within 5 years. Such a solution should be satisfactory for medical use except for the fact that the toxicity is destroyed upon freezing and no assurance can be made against the possibility that it might be frozen in shipping and handling. Some recent preliminary tests show that citrate buffers at pH 4.8 with gelatin and serum albumin make good stable solutions of the toxin at low concentrations stored at 22°C or frozen at -20°C. After three months storage the toxin at these temperatures and a concentration of 65 ng per ml showed no detectable loss. The toxin is also stable to freezing in succinate or oxalate buffers (4).

Because a lyophilized preparation seemed more practical for a wide variety of conditions we carried out lyophilization of crystalline toxin with gelatin and bovine serum albumin in phosphate buffers at pH 6.2 and 6.8. These buffers were used because freezing did not destroy the toxin. However upon lyophilization there was a certain loss in toxicity which amounted to as much as one log or 90 percent in cases, leaving only 10 percent of the toxin remaining with 90 percent detoxified toxin. Use of the

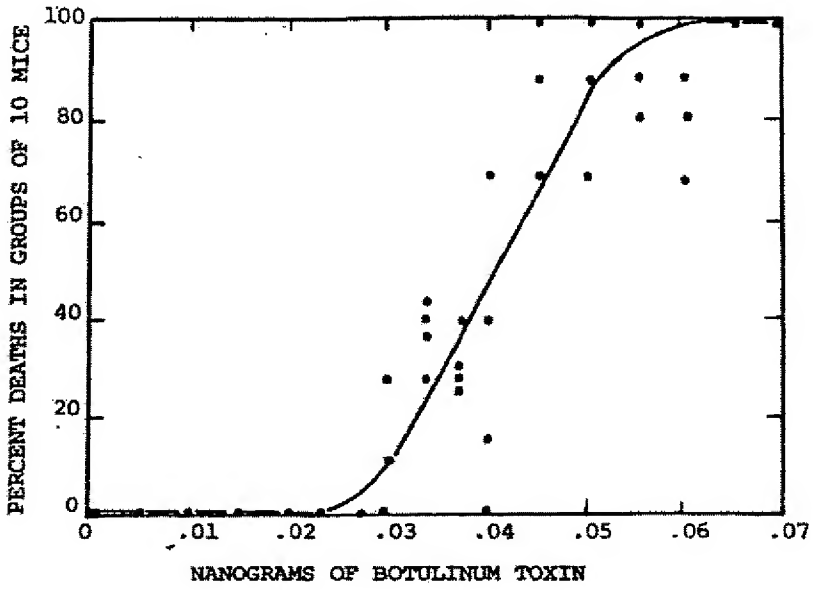

FIGURE 1. Dose response of white mice to type A botulinum toxin. Each mouse challenged intraperitoneally with the dose contained in 0.5 ml of 0.05 M sodium phosphate buffer at pH 6.2 containing 0.2% gelatin. Deaths recorded in a 96 hour period.

toxin in the phosphate-protein buffers would make a good preparation if kept frozen and used immediately after thawing. Standing at room temperature at pH 6.2 or 6.8 results in a gradual loss of toxicity. We are now investigating the use of a variety of different substances along with proteins such as some of the dextrans for stabilization of the toxin during lyophilization.

The toxicity or dose response of crystalline toxin for any medical use in humans must be determined in each particular case. However animal experimentation is indicative of the potency and nature of the toxin. The extrapolation from animal to man cannot be made directly on a weight basis, of course, but the IP dose response in mice, as illustrated in Figure 1, points out the nature of a dose response curve. These data are based on about 350 white mice weighing 18 to 22 grams to increases doses of the crystalline toxin from

0.001 to 0.065 ng contained in 0.5 ml of a 0.05 M sodium phosphate buffer with bovine serum albumin and gelatin to help stabilize the toxin at such dilute concentrations. Each dot on the chart represents the percent dead mice in a group of ten and the combined 35 dots represent the total of three separate trials with 100 to 120 mice on each trial. Some mice showed signs of botulism at about 0.01 ng up to 0.025 ng above which deaths began to appear. Those that did not die recovered within two to three weeks. A dose of 0.065 and above killed all mice in these trials. At least in our mice signs of botulism without death occurred in some mice over a 2 to 3 fold dose and the same was true over the period where death began to occur and where the dose killed all mice.

Other animals have been used. A collection of animal data of various investigators by Smith (10) indicates that 5 mouse LD_{50} will kill a 500 gram guinea pig by IP injection but 700 LD_{50} were required by the oral route. Botulism and death occurred in monkeys at 650 mouse LD_{50} per kg of body weight by the oral route. Swine are very resistant to the toxin and 20,000 mouse LD_{50} were required per kg by IV injection to cause death and 1.6×10^6 LD_{50} by the oral route. Dogs are also very resistant to the toxin. In our laboratory 20,000 mouse LD_{50} of type A toxin per kg by oral route caused no detectable signs of botulism, but 500 LD_{50} caused signs of botulism by IV injection. These dogs had no antibodies or other toxin neutralizing substances in their blood. Most of the toxin passed through the intestinal tract without absorption and was found in the feces.

One important concern is the amount of toxin to cause botulism in a person. Information on this point can only be obtained, and some has been collected, from accidental cases of poisoning. Estimates from a variety of sources (11) indicate that the dose would be between 0.1 and 1 microgram or about 3,000 to 30,000 mouse LD_{50}, but data collected by Smith (10) from various investigators over the past 60 years indicated a dose as high as 250,000 by the oral route. Most of these data are of little value for cases where the toxin would be injected because they are based on the absorption of the toxin through the alimentary tract and this amount varies greatly from person to person and from one animal species to another. Injection of the toxin and bypassing the alimentary tract makes the response much more uniform. The guinea pig appears to be the most sensitive animal to the toxin by IP injection that we know. If we assume a similar sensitivity for humans and 0.1 ng (2.5 mouse LD_{50}) was injected there would be a safety factor of more than 1000 and for one ng (25 mouse LD_{50}), the highest amount used

by Scott, there would be a safety factor of more than 1000, which is better than most drugs.

Another safety factor to be considered is the sterility of the toxin preparation. Pasteurization by heating toxin solutions buffered at pH 4.2-4.8 in the presence of gelatin or other proteins at 62°C for 30 minutes can be accomplished without detectable loss of the toxicity but heating at 80°C for one minute would destroy practically all of the toxin. Attempts at sterilization of toxin solutions by ultrafiltration in our laboratory caused a 60 percent loss in the toxicity. Sterilization by the addition of bactericidal substances may be the best approach. The two most important functions of antimicrobial preservatives in pharmaceutical products are: (a) protecting the patient from microbial contamination; and (b) preventing loss of toxicity by microbial action. According to the United States Pharmacopeia XVIII multiple dose containers must contain a suitable substance to prevent the growth of microorganisms regardless of the method of sterilization employed. Because multiple dose containers have the advantage of saving medication we are investigating the effect of some of the parabens, organic mercury compounds (thimersal) and substances like chlorohexidine on the toxin during long time storage.

Another point that should be considered here is the name of the toxin, which of course being the most lethal substance known, is scaring indeed to a patient. It is suggested that crystalline type A botulinum toxin to be used in medical practice be called OCULINUM which is derived from the words ocular and botulinum. Other bacterial products, used in medicine, have been designated by names in this manner.

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